

Multiple Influences of a Heparin-binding Growth Factor on Neuronal Development

David Schubert, Nicholas Ling, and Andrew Baird

The Salk Institute for Biological Studies, San Diego, California 92138-9216

Abstract. Heparin-binding growth factor-2 (HBGF-2; also known as basic fibroblast growth factor) is mitogenic for most anchorage-dependent cells. It is shown here that HBGF-2 stimulates cell-substratum adhesion and neurite extension in the sympathetic nerve cell line PC12. When HBGF-2 is adsorbed to artificial extracellular matrices consisting of heparin or chondroitin sulfate, it causes the formation of cellular aggregates or circles of cells, respectively. HBGF-2 is

also a nerve cell survival molecule, for it potentiates the survival of primary cultures of embryonic chick ciliary ganglion cells but not of embryonic neural retina cells. Finally, a series of synthetic peptides from the HBGF-2 sequence is described that selectively alter the biological effects of HBGF-2. The amphiphilic nature of one of these peptides is discussed with respect to its ability to stimulate cell adhesion.

A variety of molecules have been described that can alter the differentiation and survival of cultured nerve cells. These include nerve growth factor (NGF¹, 27) laminin-like molecules (7, 21, 25, 31), S100 β (24), a ciliary ganglion survival factor (5, 36), purpurin (43, 44, 46), and a 12,300-D protein that supports the survival of embryonic chick sensory neurons (6). In addition, gangliosides (35) and several other low molecular weight molecules can promote the differentiation and survival of cultured cells. To date only three of these trophic molecules have been fully sequenced: NGF (2), S100 β (24), and purpurin (Berman, P., P. Gray, E. Chen, M. LaCorbiere, F. Esch, and D. Schubert, manuscript submitted for publication).

In addition to the above trophic molecules, at least two low molecular weight heparin-binding mitogens have been purified to homogeneity from the central nervous system. The first is a 16,000-D protein with a pI of 5.8 (51) and the second is a basic 16,400-D protein (16) whose activity was initially described by Armelin (3). The primary structures of both have been recently determined (16, 17). Because these proteins are structurally distinct but similar in size and heparin-binding activity to purpurin, a nerve cell survival and adhesion molecule from the chick neural retina (46), the effect of the basic pituitary heparin-binding growth factor (the term HBGF-2 according to the nomenclature of Lobb et al. [30] is used here; also known as basic fibroblast growth factor) on nerve cell survival, adhesion, and differentiation was assayed in primary and clonal cell cultures. It will be shown that HBGF-2 causes nerve cell adhesion and differentiation, that it promotes the survival of some but not all classes of neurons, and that it induces simple social interactions be-

tween cells. Synthetic peptides derived from the HBGF-2 sequence have been developed which can alter each of these events.

Materials and Methods

Cells and Cultures

PC12 cells, clone H, were obtained from Dr. H. Hatanaka, Mitsubishi-Kasei Institute, Tokyo (20) and grown on tissue culture plastic in 10% FCS plus 5% horse serum. Neural retina tissue was separated from the pigmented epithelium of 8-d leghorn chick embryos and incubated in Hepes-buffered DME with 0.5% (wt/vol) crude trypsin (Nutritional Biochemical Corp., Cleveland, OH) for 20 min at 37°C. The cells were then rinsed three times with DME and placed in polylysine-coated tissue culture dishes with or without the indicated growth factors. The ciliary ganglion survival assay was performed as described (5, 14). Briefly, 35-mm tissue culture dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) were precoated with 2 ml of a polyornithine solution (100 μ g/ml in 0.15 M borate, pH 8.5) overnight at 4°C. The dishes were washed twice with DME and seeded with 5,000 cells from 8-d embryonic chick ciliary ganglion per dish in DME plus 10% FCS and the test compound. 24 h later cultures were assayed for viable cells either by direct visual count under phase contrast microscopy or with the use of 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (32). The data are presented as the percent of the surviving input cells.

Adhesion Assays

To assay cell-substratum adhesion, PC12 cells were metabolically labeled with [³H]leucine (5 μ Ci/ml) in DME plus serum for 15 h. The cells were washed three times with Hepes medium containing 0.3% BSA, (Calbiochem-Behring Corp., San Diego, CA), and 0.2-ml aliquots were pipetted into 35-mm Petri dishes to which HBGF-2 had been adsorbed. At the indicated times the dishes were swirled 10 times, the medium was aspirated, and the remaining attached cells were dissolved in 3% Triton X-100 and their isotope content was determined. The data are plotted as the fraction of input cells (radioactivity) that adhered at the indicated time. Variations between duplicates were <5%.

1. *Abbreviations used in this paper:* HBGF-2, heparin-binding growth factor; NGF, nerve growth factor.

HBGF-2-binding Assay

The HBGF-2 used in all experiments was purified as described by Esch et al. (16) and was a chromatographically pure 16,400-D protein. 100–150 pmol of HBGF-2 were iodinated by the lactoperoxidase method (50). The iodinated growth factor was purified by heparin–sepharose affinity chromatography and the peak of radioactivity eluting with 2 M NaCl was used in all studies. The specific activity of the iodinated growth factor was >100,000 cpm/ng. The ¹²⁵I-HBGF-2 was stored at 4°C and was stable for 2 wk.

To assay ¹²⁵I-HBGF-2 binding, PC12 cells were seeded at high density (1–2 × 10⁵ cells/well) in 48-miniwell dishes that had been precoated with polylysine. The following day the cells were washed twice with binding buffer (F12 medium, 25 mM Hepes, pH 7.5, 0.2% gelatin) and incubated in 200 μl of the same buffer. ¹²⁵I-HBGF-2 was then added to the cells in a total volume of 10 μl. Both total and nonspecific binding were determined in quadruplicate or sextuplet wells. The cells were incubated for the indicated times at 4°C and the reaction was terminated by three washes with binding buffer. Cells were solubilized with 200 μl of 1% Triton X-100 in water and an aliquot was removed and counted on a γ-counter. Nonspecific binding to the cells, as determined by the inhibition of ¹²⁵I binding by 4 μg of HBGF-2, was <20% of the total binding.

Preparation of Artificial Matrix

An artificial matrix was created to assay the ability of substratum bound HBGF-2 to influence the morphological differentiation of PC12 cells. Poly-L-lysine (Sigma Chemical Co., St. Louis, MO) was incubated at 50 μg/ml overnight in 35-mm tissue culture dishes (Falcon Labware). The dishes were washed twice with water and incubated overnight with either heparin (porcine intestinal mucosa, Grade 1, Sigma Chemical Co.) or chondroitin sulfate A (Miles Laboratories, Inc., Elkhart, IN) at 200 μg/ml. The following day the dishes were washed twice with water and incubated in Hepes-

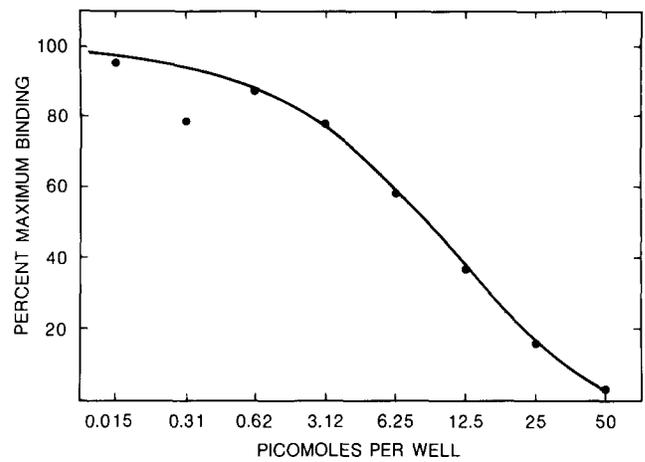


Figure 1. Displacement of cell-associated ¹²⁵I-HBGF-2 by pituitary HBGF-2. PC12 cells were incubated with 4 ng/ml of ¹²⁵I-HBGF-2 and increasing concentrations of unlabeled HBGF-2 for 2 h at 4°C. The binding was performed as described in Materials and Methods. 10% of the input ¹²⁵I-HBGF-2 bound to the cells in the absence of other peptides.

buffered DME overnight with 1 pmol/dish of HBGF-2. Greater than 95% of the input HBGF-2 bound to each surface as determined by the inclusion of ¹²⁵I-HBGF-2. Finally, the dishes were washed twice with DME and the cells incubated on these surfaces in serum-free N₂ medium (10).

Table I. Synthetic Fragments of HBGF-2

Peptide	Fragment	Sequence	Peptide	Charge	Inhibition of HBGF-2 binding %	Stimulation of adhesion %
A	[Tyr ¹⁰] (1–10)OH	PALPEDGGSY	A	–2	NS	40
B	[Tyr ⁵⁰] (30–50)NH ₂	FFLRIHPDGRVDGVREKSDPY	B	+1	11	NS
C	[Tyr ^{69,87}] (69–87)NH ₂	YANRYLAMKEDGRLLASKY	C	+3	NS	NS
D	(106–115)NH ₂	YRSRKYSSWY	D	+4	60	80
E	(1–24)NH ₂	PALPEDGGSGAFPPGHFKDPKRLY	E	+1	12	230
F	(24–68)NH ₂	YCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQ AEERGVVSIKGV	F	+3 A	60	450
F ¹	[Tyr ²⁵] (25–68)NH ₂	YKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQA AEERGVVSIKGV	F ¹	+3 A	95	450
G	[Tyr ⁸⁷] (73–87)NH ₂	YLAMKEDGRLLASKY	G	+2	NS	NS
H	(121–146)NH ₂	TGQYKLGPKTGPGQKAILFLPMSAKS	H	+5 A	NS	NS
I	(16–24)NH ₂	HFKDPKRLY	I	+3	12	20
J	(36–39)NH ₂	PDGR	J	+1	NS	–
K	(30–39)NH ₂	FFLRIHPDGR	K	+2	6	NS
K ¹	(32–39)NH ₂	LRIHPDGR	K ¹	+2	5	NS
M	(32–53)NH ₂	LRIHPDGRVDGVREKSDPHIKL	M	+2	NS	NS
N	(93–120)NH ₂	FFFERLESNNYNTYRSRKYSSWYVALKR	N	+5	82	480
N ¹	(103–120)NH ₂	YNTYRSRKYSSWYVALKR	N ¹	+6	80	460

Complete sequence:

10 20 30 40 50 60 70 80
 PALPEDGGSG AFPPGHFKDP KRLYCKNGGF FLRIHPDGRV DGVREKSDPH IKLQLQAEER GVVSIKGVCA NRYLAMKEDG
 90 100 110 120 130 140 146
 RLLASKCVTD EFFFFERLES NNYNTYRSRK YSSWYVALKR TGQYKLGPKT GPGQKAILFL PMSAKS

Peptides were synthesized as described in Materials and Methods and tested for their ability to block HBGF-2 binding and to stimulate adhesion. To determine the effect of the peptides on HBGF-2 binding, PC12 cells were incubated for 10 min at 4°C in the presence of the indicated synthetic peptides, followed by the addition of ¹²⁵I-HBGF-2. The amount of HBGF-2 binding was determined as in Materials and Methods and the data represented as the percent inhibition of binding by the peptides relative to the control cultures. Peptides were used at 100 μg/well. To assay the stimulation of cellular adhesion by synthetic peptide fragments of HBGF-2, the indicated peptides were adsorbed to plastic petri dishes at 300 μg/35-mm culture dish. All of the input peptides, except J, adhered to the plastic as determined by HPLC or protein analysis. The adhesion of input cells after 1-h incubation was determined as in Fig. 2 and presented as the percent stimulation relative to neutral petri dish surfaces where 6–8% of the input cells adhered. All experiments were repeated at least three times with similar results. NS, not significant. A, amphiphilic.

Peptide Synthesis

All HBGF-2 peptides were prepared by solid phase methods (34) using the methylbenzhydrylamine resin. The exception was the [Tyr¹⁰] (1-10)OH fragment that was made with a N^o-Boc-(2,6-dichlorobenzyl) tyrosine coupled chloromethyl resin (28). The synthetic products were purified by gel filtration, ion-exchange, and partition chromatography as described elsewhere (29). Fragments F and F¹ were also purified by preparative HPLC. The purified products had the correct amino acid composition and the purity ranged from 90 to 98% by HPLC analysis.

Results

HBGF-2 Binding to Cells

HBGF-2 is mitogenic for essentially all of the anchorage-dependent cultured cells on which it has been tested, suggesting that it interacts with a relatively common cell surface receptor. To determine if HBGF-2 can bind to nerve cells, HBGF-2 was labeled with ¹²⁵I and its ability to bind to the NGF-responsive PC12 cell line assayed. ¹²⁵I-HBGF-2 binds to PC12 cells in a time dependent manner, with maximum binding occurring at 2 h. Fig. 1 shows that the binding of ¹²⁵I-HBGF-2 is inhibited by the unlabeled molecule with 50% displacement at ~7 pmol/well (3.5×10^{-8} M). The iodinated HBGF-2 is as mitogenic as the native molecule for endothelial cells. Neither NGF nor the heparin binding adhesion molecule N-CAM (12) competes with HBGF-2 for binding to the cell surface when tested at 5 μ g/ml.

Since HBGF-2 has been recently sequenced (16), it is possible to synthesize peptide fragments that cover the complete primary structure of the molecule and to ask if any of these synthetic peptides can alter the cell binding or biological activity of HBGF-2. In this manner it may be possible to define which parts of the molecule are responsible for its diverse actions. Table I shows the structure of HBGF-2 and the synthetic peptides, which are identified by a letter code. The peptides with a net charge are indicated and the amphiphilic or β -sheet (alternating hydrophobic and hydrophilic residues) sequences marked by the letter A. There are two amphiphilic stretches of amino acids (residues 34-41 and 49-58) in peptide F and one sequence (residues 123-133) in peptide H toward the COOH-terminus.

To determine which parts of the HBGF-2 primary structure are involved in cell binding, the ability of each peptide to block ¹²⁵I-HBGF-2 binding was determined. Peptides were used at a concentration of 100 μ g/ml or $\sim 5 \times 10^{-5}$ M for a 20-residue peptide. Table I shows that only peptides D (residues 106-115), F and F¹ (residues 24-68), and N and N¹ (residues 93, 103-120) are very effective in inhibiting the HBGF-2-cell interaction. It is shown elsewhere that these peptides interact strongly with heparin (4); heparin can also block the binding of HBGF-2 to cells at a concentration of 1 μ g/ml. These results suggest that the HBGF-2-cell surface interaction may require an interaction of the growth factor with a cell surface heparin or heparan sulfate proteoglycan.

PC12 Cell-Substratum Adhesion

Cell-substratum adhesiveness plays a critical role in the initiation of neurite outgrowth in many types of cultured nerve cells and on the rate of neurite outgrowth of all cultured nerve cells (see, for example, 26, 41, 42). To determine if substratum-bound HBGF-2 alters the adhesiveness of PC12 cells to an adhesive neutral surface, low density exponentially dividing PC12 cells were labeled overnight with [³H]leucine, plated onto petri dish surfaces that had been coated with 30 pmol of HBGF-2, and the percent of input cells that adhered to the HBGF-2 substratum were assayed as a function of time. Fig. 2A shows that HBGF-2 stimulates the adhesion of PC12 cells relative to the petri dish surface alone. The concentration dependence of this adhesion is shown in Fig. 2B. The half maximal rate of adhesion to HBGF-2 is at a HBGF-2 concentration of 10 pmol/35-mm petri dish. The glycosaminoglycans heparin and heparan sulfate block the adhesion of PC12 cells to HBGF-2, but chondroitin sulfate is ineffective. There is some specificity with respect to the ability of heparin to inhibit adhesion, for heparin is only marginally effective in inhibiting the adhesion of PC12 cells to laminin and fibronectin (1; Schubert, D., unpublished observation). HBGF-2 is therefore an adhesion protein for nerve cells and it is effective in causing adhesion in the picomole range.

To understand which parts of the primary sequence of HBGF-2 are responsible for promoting the HBGF-2-cell in-

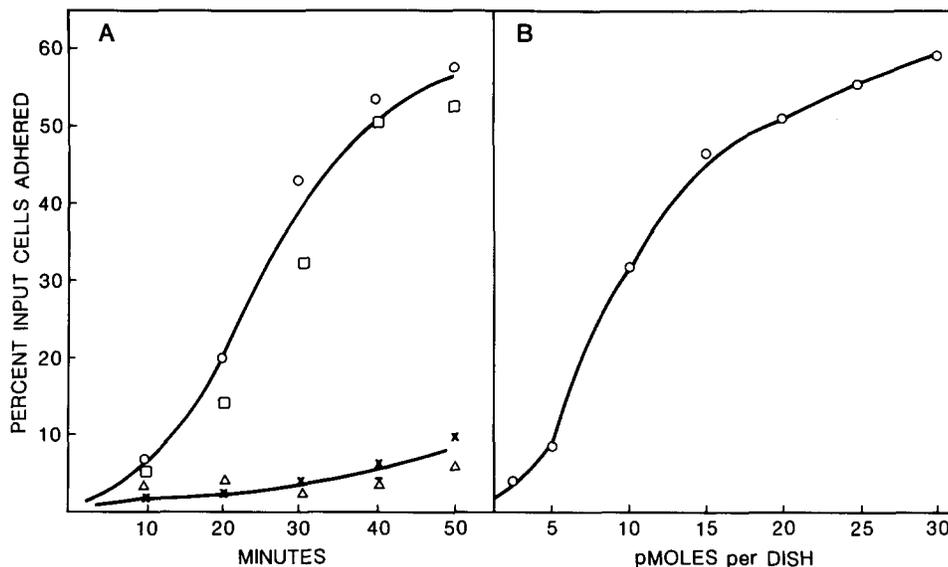


Figure 2. Stimulation of cell-substratum adhesion by HBGF-2. The adhesion of isotopically labeled PC12 cells to HBGF-2-coated surfaces was determined as described in Materials and Methods. (A) Adhesion of PC12 cells to substrata containing 30 pmol of HBGF-2 as a function of time. (X) Petri dish alone; (open circles) HBGF-2; (open squares) HBGF-2 plus 200 μ g/ml of chondroitin sulfate; (open triangles) HBGF-2 plus 200 μ g/ml of heparan sulfate. (B) Concentration dependence of adhesion. Petri dishes were coated with the indicated concentrations of HBGF-2 and the percent of input cells adhered determined after 1 h.

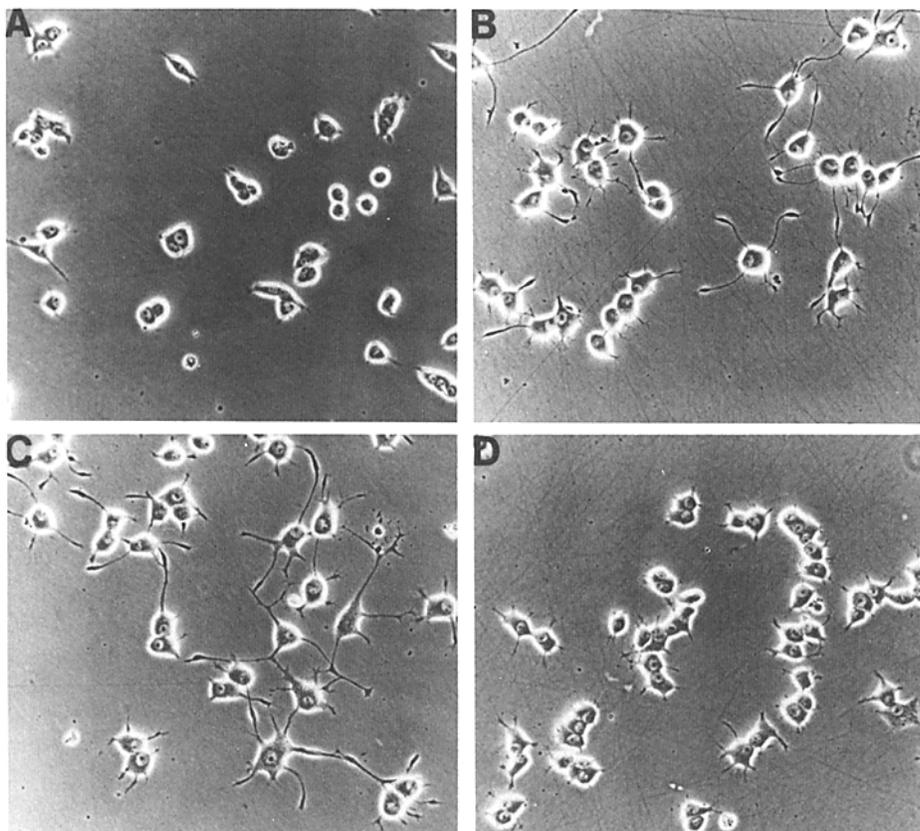


Figure 3. Effect of HBGF-2 on PC12 neurite outgrowth. PC12 cells were plated on polylysine-coated surfaces in serum-free N_2 medium as described in Materials and Methods. The phase photomicrographs were taken 3 d after the addition of the growth factors. (A) Control. (B) β -NGF at 10 ng/ml. (C) HBGF-2 at 50 pM. (D) HBGF-2 plus 100 μ g/ml of peptide F (24–68).

teraction, 100 μ g/ml of each synthetic peptide (Table I) was added to the culture dishes containing HBGF-2 bound to the substratum before the addition of cells. Of the 16 peptides tested, none blocked the adhesion of PC12 cells to HBGF-2. However, several did slightly stimulate the rate of adhesion over a period of 1 h. Since it is possible that the synthetic peptides adhere to the substratum during the course of the assay and promote cell-substratum adhesion rather than block it, the longer synthetic peptides were adsorbed to the surface of petri dishes and assayed for their ability to stimulate cell adhesion. Table I shows that the amphiphilic 24–68 sequence (peptides F and F¹) and the peptide sequence between residues 93 and 120 (peptide N) are very effective at stimulating cell-substratum adhesion. Charge and amphiphilicity alone is not, however, responsible for the stimulation of adhesion, for the highly positively charged and amphiphilic peptide H (residues 121–146) does not stimulate adhesion. Except for the NH_2 -terminal (residues 1–24) peptide, the other peptides are ineffective. These data suggest that although peptides F and N block the binding of HBGF-2 to cells, they do not interfere with substratum-bound HBGF-2-cell adhesion because they are rapidly adsorbed to the substratum themselves and promote adhesion.

Morphological Differentiation of PC12 Cells

When NGF is added to exponentially dividing PC12 cells, they respond by the extension of neurites (Fig. 3 B). PC12 cells also respond to HBGF-2 by extending neurites (Fig. 3 C). The concentration of HBGF-2 required to give the maximum rate of neurite extension is \sim 25 pM (Table II), and there is no alteration in the response if concentrations 50-fold

higher are used. Neurite outgrowth continues for several days in the presence of HBGF-2 and, as with NGF, is reversible when the growth factor is removed. At lower concentrations of HBGF-2 neurite outgrowth ceases after a few days and the neurites retract (Table II). Repeated addition of HBGF-2 allows neurite outgrowth to continue for up to a week and it ceases only when the cell density becomes high, leading to cell detachment.

To determine which part of the HBGF-2 molecule is responsible for the induced neurite outgrowth, the synthetic peptides were examined for their ability to block HBGF-2 induced neurite outgrowth. Only peptides F and F¹ (Table I)

Table II. HBGF Titration on Neurite Outgrowth

HBGF	Days				
	1	2	3	4	5
<i>pM</i>					
500	+	+	+	+	+
250	+	+	+	+	+
100	+	+	+	+	+
50	+	+	+	+	–
25	+	+	+	–	–
10	+	+	–	–	–
5	+	+	–	–	–
2.5	+	–	–	–	–
1	–	–	–	–	–

Cells were plated on polylysine-coated dishes in N_2 medium and HBGF added at the indicated concentrations. The presence of neurites was scored on the following days in at least 200 cells. A plus sign indicates that at least half of the cells within population had neurites >1 cell diameter (20 μ m).

of residues 24–68 block the HBGF-2-induced neurite outgrowth (Fig. 3 D). None of the other synthetic peptides has any obvious effect on the HBGF-2-induced morphological differentiation of the PC12 cells when tested at concentrations up to 200 $\mu\text{g}/\text{ml}$, nor do any of the peptides cause neurite outgrowth when added directly to the culture medium or when adsorbed to the culture dish substratum before plating of the cells.

HBGF-2-induced neurite formation is completely blocked by 100 $\mu\text{g}/\text{ml}$ of heparin but not by chondroitin sulfate. Neurite formation induced by NGF on polylysine surfaces is also reduced in the presence of 100 $\mu\text{g}/\text{ml}$ heparin or heparan sulfate but not completely eliminated as it is in the case of HBGF-2. In both cases the cells round up and become more phase bright, but neurites are still present in over 70% of the cells grown in NGF but in <5% of cells with HBGF-2. The amphiphilic peptide F (residues 24–68) does not block NGF-induced neurite outgrowth.

HBGF-2-induced Supracellular Assemblies

Basement membranes can dramatically effect the morphological differentiation of a tissue (see, e.g., reference 9). If culture conditions could be devised where basement membrane-like structures influence the type of cell-cell interactions seen in vivo, then it may be possible to gain some insight into the chemistry of the cell-basement membrane interactions that are responsible for these events. Since HBGF-2 can bind heparin it is probable that it is associated with heparin-rich basement membranes and therefore may influence cells that interact with that membrane. To construct artificial basement membranes on the substrata of culture dishes, tissue culture dishes were first coated with poly-L-lysine and then with either chondroitin sulfate or heparin. HBGF-2 was added to some of the dishes overnight and the dishes were washed extensively. The effect of these different substrata on the cells was then assayed. Fig. 4 shows that in the absence of HBGF-2, cells on both heparin (Fig. 4 A) and

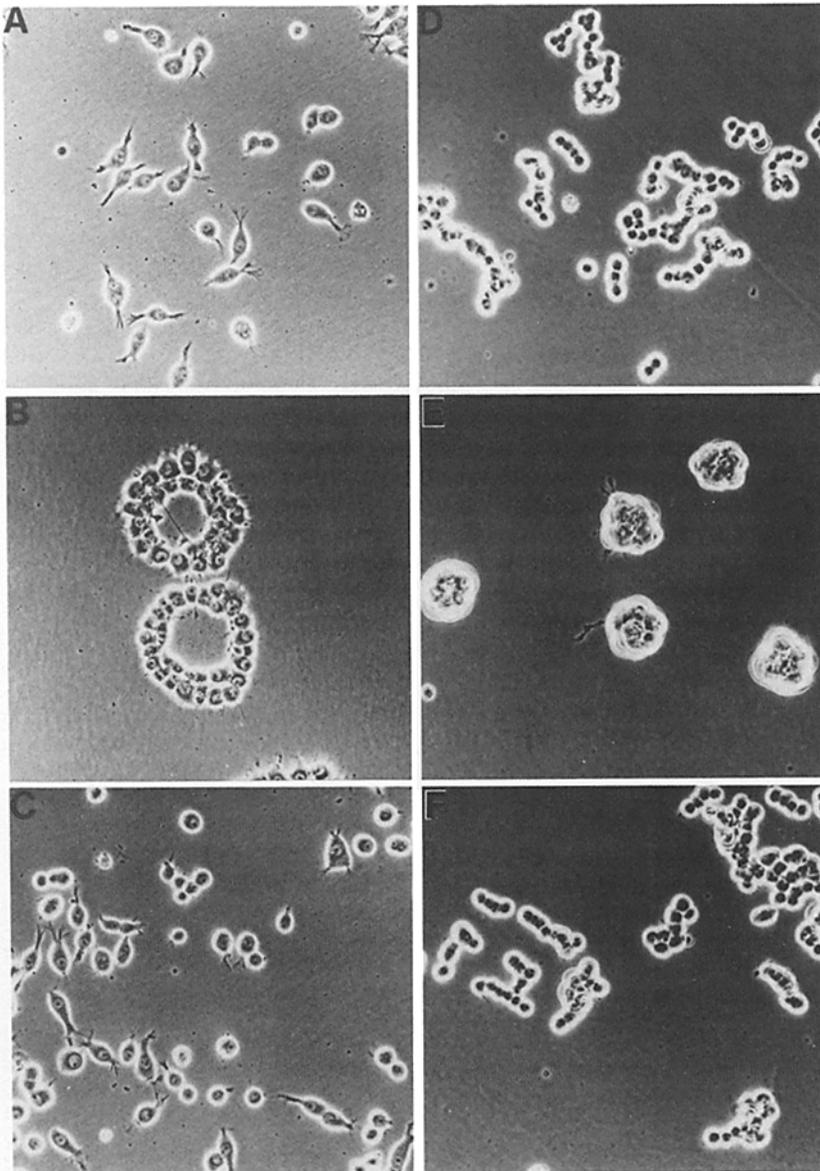


Figure 4. Effect of artificial matrices on cellular interactions. PC12 cells were plated upon the surfaces indicated below at 1×10^5 cells/35-mm culture dish and photographed 4 d later. The cell diameter is $\sim 20 \mu\text{m}$. These experiments have been repeated at least 12 times with similar results. A detailed kinetic and ultrastructural analysis of circle formation will be presented elsewhere. (A) Chondroitin sulfate. (B) Chondroitin sulfate plus HBGF-2. (C) Chondroitin sulfate plus HBGF-2 plus 100 $\mu\text{g}/\text{ml}$ of peptide F. (D) Heparan sulfate. (E) Heparan sulfate plus HBGF-2. (F) Heparan sulfate plus HBGF-2 plus peptide F.

chondroitin sulfate (Fig. 4 *D*) have a round, phase-bright morphology, and that the cells on heparin tend to form chains. However, in the presence of substrate-bound HBGF-2 or HBGF-2 added to the dishes directly, the arrangement of the cells on the culture dish changes dramatically. Cells plated on a matrix of chondroitin sulfate and HBGF-2 form circles consisting of 10–30 cells (Fig. 4 *B*). This process apparently takes place via the movement of cells over the substratum since the cells are randomly distributed over the dish 5 h after plating. HBGF-2 is only slightly mitogenic for PC12 cells ($5 \pm 2\%$ increase in cell number over control after 3 d). In contrast to cells plated on chondroitin sulfate and HBGF-2, cells on the heparin HBGF-2 matrix form tight aggregates that are loosely attached to the substratum (Fig. 4 *E*). NGF adsorbed to these substrata causes the formation of some circles on chondroitin sulfate and modest neurite outgrowth. NGF on heparin causes very few aggregates to form. Although NGF produces changes in cellular behavior similar to those caused by HBGF-2, the latter is much more effective.

Since some of the synthetic peptides that influence HBGF-2 binding also block the morphological differentiation of PC12 cells by HBGF-2, the peptides were assayed for their ability to inhibit the morphological changes caused by HBGF-2 bound to the two glycosaminoglycans. Of the peptides tested (Table I) only the amphipathic 24–68 sequence (peptide F) blocks both circle formation on chondroitin sulfate and aggregate formation on heparin (Fig. 4, *C* and *F*). Peptide N (residues 93–120) causes the cells to become very flat, and, in the presence of soluble HBGF-2, grow long neurites, but it does not block HBGF-2-induced formation of circles on chondroitin sulfate or aggregate formation on heparin.

Cell Viability

Two populations of embryonic nerve cells were used to assay

the effect of HBGF-2 on cell viability: 8-d embryonic chick ciliary ganglion and neural retina cells. The embryonic ganglion cells normally die within 24 h in the absence of added trophic factors (see, e.g., references 14, 32) while the viability of the embryonic neural retina cells declines with a half-life of 3 d (43). To test the effect of HBGF-2 on the survival of both cell populations, the cells were dissociated and plated in DME containing either 10% FCS (ciliary ganglion) or serum-free medium (neural retina cells) in the presence or absence of HBGF-2. Viable cell number was determined at 24 h (ciliary ganglion) or at 1-d intervals (neural retina cells). Fig. 5 *A* shows that HBGF-2 did not alter the survival rate of dissociated neural retina cells. Purpurin, a cell survival molecule for neural retina cells, was added to some dishes as a positive control. Fig. 5 *B* shows that HBGF-2 supported the survival of 8-d embryonic ciliary ganglion cells at a half maximal effectiveness of ~ 5 pM HBGF-2. These data show that HBGF-2 affects the survival of dissociated ciliary ganglion cells but not that of neural retina cells from the same age embryo. When the ability of all the synthetic peptides to block the effect of HBGF-2 on ciliary cell survival was examined, only the amphiphilic HBGF-2 peptide F of residues 24–68 was effective.

Discussion

The above data show that HBGF-2 has several diverse effects on nerve cells in culture. These include increasing the rate of cell substratum adhesion (Fig. 2), the stimulation of neurite formation (Fig. 3), increased cell survival of ciliary nerve cells in primary cultures (Fig. 5), and, when adsorbed to artificial extracellular matrices, the ability to cause the formation of circles of cells or the formation of cell aggregates, depending upon the composition of the matrices (Fig. 4). When a series of synthetic peptides derived from the HBGF-2 sequence were examined for their ability to block its biological effects, an amphiphilic peptide of HBGF-

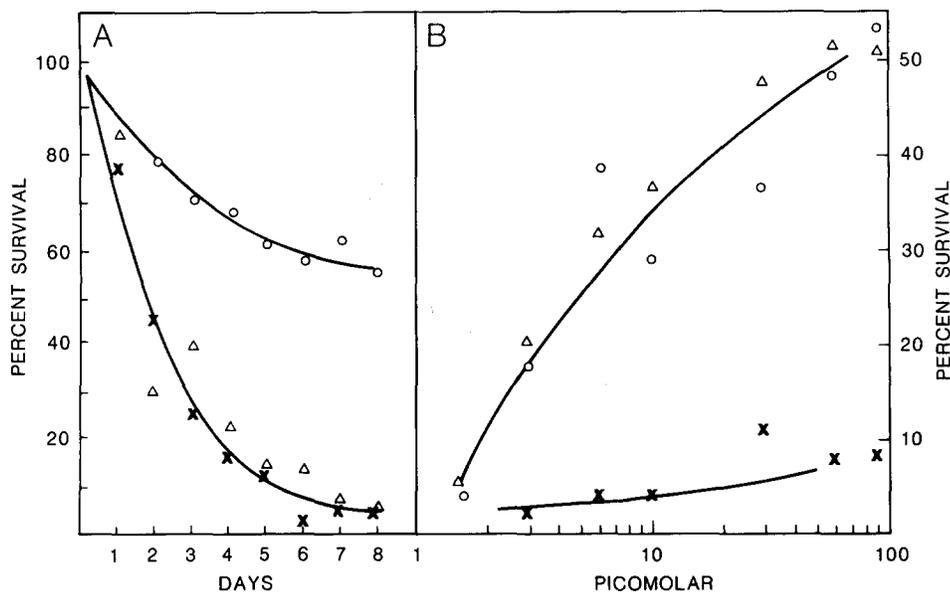


Figure 5. Effect of HBGF-2 on ciliary ganglion and neural retina survival. (*A*) Neural retina cells were dissociated from 8-d embryos, plated at 2×10^4 cells/35-mm tissue culture dish coated with polylysine, and the viable cell number determined daily as described in Materials and Methods. Concentrations of HBGF-2 up to 20 nM were tested without effect. As a positive control purpurin was added at 100 ng/dish. (open triangles) Control no additive; (X) 100 pM HBGF-2; (open circles) purpurin. (*B*) Ciliary ganglion cells were dissociated from 8-d embryos as described in Materials and Methods. The number of viable cells as determined 20 h after plating in the indicated amounts of HBGF-2. (open circles and open triangles) Two independent experiments with HBGF-2 (X) 10 pM HBGF-2 plus 100 μ g/ml of peptide F. Similar results were obtained with F¹.

2, residues 24–68, blocked HBGF-2-induced neurite outgrowth, HBGF-2-induced circle and aggregate formation, and the HBGF-2-dependent survival of ciliary cells. This peptide and several others were able to stimulate cell-substratum adhesion.

Several other proteins have been described that prolong the survival of neurons in culture. The best studied are NGF (27), a 12,300-D protein from pig brain (6), and a ciliary ganglion neurotrophic factor (5) that is similar if not identical to purpurin, a 20,000-D protein that inhibits the death in culture of both embryonic neural retina and ciliary ganglion cells (43, 46). HBGF-2 is a protein mitogen for most anchorage-dependent primary cultures (4) and there has been a recent report that it increases the survival of cells in primary cultures of rat hippocampus (53). Fig. 5 shows that HBGF-2 has the ability to promote the survival of cultured embryonic chick ciliary ganglion cells but not chick neural retina cells. In contrast purpurin, an eye derived molecule, promotes the survival of both populations (46). HBGF-2 therefore shows some cell-type specificity with respect to enhancing nerve cell survival. Both HBGF-2 and purpurin increase cell-substratum adhesion and laminin, an exceptionally potent adhesion promoting molecule, also causes nerve cell survival (7).

Numerous studies have shown that there is a relationship between cellular adhesion and division, and it is likely that these observations can be extended to cell survival. Folkman and Moscona (19) showed that small changes in cell shape, which is directly influenced by cell-substratum adhesion, have large effects upon the probabilities of initiating DNA synthesis. The more spread a cell the greater the likelihood of DNA synthesis (see also 37). There is also a correlation between cell shape and physiology. When cells that are anchorage dependent for growth (49) are placed in suspension culture they rapidly become arrested in the G₁ phase of the cell cycle (38) and RNA and protein synthesis is rapidly decreased (8, 18). They eventually die (18, 19). It has been argued that these phenotypic changes may be due to differences in nutrient transport between more and less spread cells (22). Since (a) HBGF-2 only stimulates mitosis in substrate attached cells, (b) HBGF-2 does not stimulate DNA synthesis when a HBGF-2-responsive cell line is cultured in suspension (47), and (c) HBGF-2 promotes the adhesion of cells (Fig. 2), it follows that one of the mechanisms that mediates the effects of HBGF-2 may be to increase cell-substratum adhesion and therefore cell spreading. The efficiency of this interaction is dictated by the composition of the extracellular matrix.

Components of the extracellular matrix can influence the migration and differentiation of juxtapositioned cells (see reference 9 for a review). Within the nervous system the role of extracellular matrix components such as heparan sulfate is only recently becoming appreciated. In addition to HBGF-2, several other molecules bind specifically to heparin and heparan sulfate. These include purpurin (46), N-CAM (12, 13), the well characterized basement membrane component laminin (7, 15), and fibronectin (11). The heparin binding fragment of laminin alone promotes nerve cell survival and neurite outgrowth (15). A neurite promoting activity, thought to be laminin, has also been isolated from an extracellular macromolecular complex containing heparan sulfate proteoglycan (25). In addition myelination is initiated by

Schwann cell interactions with a nerve cell surface heparan sulfate proteoglycan (39). These data suggest that the interactions between the extracellular environment and cell surface heparan sulfate proteoglycans have profound effects within the developing nervous system. Since most of the molecules that can bind heparin and heparan sulfate are released from cells, it is likely that they are localized within the matrix. Thus one of the functions of heparin binding may be to keep the secreted molecule near its cell of origin via entrapment in the matrix. Once in the matrix molecules such as HBGF-2 may support cell adhesion, cell survival, and direct neurite outgrowth. Upon release from the matrix they become potent mitogens.

It has been reported elsewhere that HBGF-2 causes neurite extension in PC12 cells and that there is spontaneous retraction of neurites after 6 d, even in the continued presence of HBGF-2 (52). In these experiments, substantially greater concentrations of HBGF-2 were required to cause neurite outgrowth than those reported here and there was no indication of the purity of their protein. Since we do not observe that the neurites retract in the continued presence of HBGF-2 except when the cultures became very dense, it is possible that contaminants in the HBGF-2 preparations of Togari et al. (52) were adversely affecting the cells. In addition, the cultures described here were maintained in polylysine-coated substrata and serum-free N₂ media as opposed to serum-containing media. Serum tends to reduce cell-substratum adhesion and cause neurite retraction (45). It is clear, however, that while NGF and HBGF-2 both affect neurite outgrowth in PC12 cells, their effects are not identical. There are, for example, differences in neurite morphology between HBGF-2- and NGF-induced cells, for those caused by HBGF-2 are more straight than those made by NGF (Fig. 3). Moreover, the maximum rate of neurite outgrowth and neurite arborization are somewhat lower in HBGF-2-treated cultures relative to those exposed to NGF. It is likely that NGF and HBGF-2 share a subset of events that leads to neurite elongation, but that not all of the chemistry involved is identical. As suggested before (52), this situation is analogous to that with cAMP, which can mimic the short term effects of NGF but not all of its long term effects (42).

The experiments showing that HBGF-2 can affect cell-cell interactions in culture (Fig. 4) suggest that it may play a role in morphogenesis. Although the mechanisms involved in the formation of circles of cells or cellular aggregates are not known, it is likely that they involve the ability of HBGF-2 to modulate the adhesive properties of cells to the substratum. As initially outlined by Steinberg (48) in his differential adhesion hypothesis, changes in the relative adhesiveness of cells to each other and to their environment can have profound effects on the topological distribution of cells within a "closed" system. The combination of HBGF-2 and glycosaminoglycans must influence the adhesiveness of the PC12 cells such that on heparin they are less adhesive to the substratum than to themselves (aggregate formation), on the glycosaminoglycans alone they adhere more tightly to the substrata (single attached cells), and on the combination of chondroitin sulfate and HBGF-2 they have an intermediate adhesiveness to each other and the substratum (circle formation).

Of the 16 synthetic peptides that cover the entire sequence of the HBGF-2 molecule, only peptides D (residues 106–

115), F (residues 24–68), and N (residues 93–120) inhibit the binding of ^{125}I -HBGF-2 to cells (Table I), while peptides F, N, and E (NH₂-terminal 24 residues) stimulate cell-substratum adhesion (Table I). All of these peptides have a net positive charge at neutrality and peptide F has two internal amphiphilic or β -sheet sequences. An amphiphilic or β -sheet sequence is one that causes a segregation of hydrophobic and hydrophilic residues when the secondary structure is formed. This can occur when the peptide has alternating hydrophobic and hydrophilic residues. Such peptides can form stable β -sheet structures so that the hydrophobic amino acids project above and the hydrophilic below the plane of the peptide. Amphiphilic β -sheets should interact strongly with cellular membranes, for the hydrophobic residues should interact with the hydrophobic aspect of the membrane, whereas the hydrophilic residues should interact with the cell surface glycoproteins or aqueous environment. Peptide hormones with amphiphilic secondary structures interact strongly with cell membranes and this interaction is critical for their biological activities (23). A study of both natural and synthetic peptides suggested that most amphiphilic peptides with a net charge of $>+2$ interact with cells and promote adhesion (40). In contrast the common polycations such as polylysine and polyornithine must have a net charge of $+10$ or more to mediate adhesion (33). The data presented here are only in partial agreement with these conclusions. Of the three HBGF-2 sequences that block ^{125}I -HBGF-2 binding to cells or promote cell-substratum adhesion when adsorbed to a plastic surface, only one contains amphiphilic sequences; two have a net charge of $>+2$. The NH₂-terminal peptide E, residues 1–24, has a net charge of $+1$ and no alternating hydrophobic and hydrophilic residues. It promotes cell adhesion but is relatively ineffective in blocking HBGF-2 binding. Peptide F (residues 24–68) contains two amphiphilic sequences, Leu-Arg-Ile-His-Pro-Asp and Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu, and has a net charge of $+3$. The last biologically active peptide N (residues 93–120) has a charge of $+5$, but no amphiphilic sequence. However, neither charge nor amphiphilic secondary structure, nor the combination of the two is sufficient to cause adhesion. Peptide H (residues 121–146) has a net charge of $+5$ and an amphiphilic series of residues, Gln-Tyr-Lys-Leu-Gly-Pro-Lys and Gly-Pro-Gly, but it is totally inactive in promoting cell-substratum adhesion or blocking the binding of HBGF-2 to cells. These data suggest that peptides F and N have unique sequences that allow them to both block HBGF-2 binding and promote cellular adhesion. The receptors for these sequences must be on many cell types for they stimulate the adhesion of most cell types (unpublished observation). This is not surprising since a HBGF-2 is mitogenic for most cell types in primary culture. How is it possible, however, that peptides F and N both block HBGF-2 binding to cells but that only peptide F can block the biological responses of cells to HBGF-2? It is probable that peptide N is either more rapidly degraded or has a relatively low affinity for the cell surface HBGF-2 binding site. It may also adsorb nonspecifically to the surface of cells or the substratum during the long term (1–2 d) duration of the biological assays and therefore not compete effectively with the higher affinity binding of HBGF-2. In contrast, the ^{125}I -HBGF-2 binding assay only takes 2 h and the nonspecific adsorption may be relatively less. Clearly more work is required to understand the details of the HBGF-2-cell in-

teraction, but the use of the synthetic peptides described above should allow for a better understanding of this interaction at the cell surface. These peptides should also prove useful in vivo for elucidating the possible role of HBGF-2 in neuronal development.

We thank Drs. T. Hunter, S. Haleboua, P. Maher, and M. LaCorbiere for their comments on the manuscript and Nancy Goodknight for its preparation. We are also indebted to Dr. Marston Manthorpe for help with the ciliary ganglion survival assay and Dr. H. Hatanaka for the PC12 cell line.

This work was supported by grants from the National Institutes of Health.

Received for publication 1 October 1986, and in revised form 20 November 1986.

References

- Akeson, R., and S. L. Warren. 1986. PC12 adhesion and neurite formation on selected substrates are inhibited by some glycosaminoglycans and a fibronectin-derived tetrapeptide. *Exp. Cell Res.* 162:347–362.
- Angeletti, R. H., and R. Bradshaw. 1971. Nerve growth factor from mouse submaxillary gland: amino acid sequence. *Proc. Natl. Acad. Sci. USA.* 68:2417–2420.
- Armelin, H. A. 1973. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proc. Natl. Acad. Sci. USA.* 70:2702–2706.
- Baird, A., F. Esch, P. Mormede, N. Ueno, N. Ling, P. Bohlen, S. Ying, W. Wehrenberg, and R. Guillemin. 1986. Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. *Recent Prog. Horm. Res.* 42:143–205.
- Barbin, G., M. Manthorpe, and S. Varon. 1984. Purification of the eye ciliary neurotrophic factor. *J. Neurochem.* 43:1468–1478.
- Barde, Y. A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:549–553.
- Baron van Evercooren, A., H. K. Kleinmann, S. Ohno, P. Marangos, J. P. Schwartz, and M. E. Dubois-Dalq. 1982. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* 8:179–193.
- Ben-Zeev, A., S. R. Farmer, and S. Penman. 1980. Protein synthesis requires cell-surface contact while nuclear events respond to cell shape in anchorage dependent fibroblasts. *Cell.* 21:365–372.
- Bissell, M. J., H. G. Hall, and G. Parry. 1982. How does the extracellular matrix direct gene expression? *J. Theor. Biol.* 99:31–68.
- Bottenstein, J., and G. Sato. 1979. Growth of a rat neuroblastoma cell line in serum free supplemented media. *Proc. Natl. Acad. Sci. USA.* 76:514–517.
- Carnonetto, S., M. M. Gruver, and D. C. Turner. 1983. Nerve fiber growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. *J. Neurosci.* 3:2324–2335.
- Cole, G. J., and L. Glaser. 1986. A heparin-binding domain from N-CAM is involved in neural cell-substratum adhesion. *J. Cell Biol.* 102:403–412.
- Cole, G. J., D. Schubert, and L. Glaser. 1985. Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. *J. Cell Biol.* 100:1192–1199.
- Collins, F. 1985. Electrophoresis similarity of the ciliary ganglion survival factors from different tissues and species. *Dev. Biol.* 109:255–258.
- Edgar, D., R. Timpl, and H. Thoenen. 1984. The heparin binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1463–1468.
- Esch, F., A. Baird, N. Ling, N. Ueno, F. Hill, L. Denoroy, R. Klepper, F. Gospaderovicz, P. Bohlen, and R. Guillemin. 1985. Primary structure of bovine pituitary basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 82:6507–6511.
- Esch, F., N. Ueno, A. Baird, F. Hill, L. Denoroy, N. Ling, F. Gospaderovicz, and R. Guillemin. 1985. Primary structure of bovine brain acidic fibroblast growth factor (FGF). *Biochem. Biophys. Res. Commun.* 133:554–562.
- Farmer, S. R., A. Ben-Zeev, B. J. Benoke, and S. B. Penman. 1978. Altered translatability of mRNA from suspended anchorage-dependent fibroblasts: reversal upon cell attachment to a surface. *Cell.* 15:627–637.
- Folkman, J., and J. Moscona. 1978. The role of cell shape in growth control. *Nature (Lond.)* 273:345–349.
- Hatanaka, H. 1983. Nerve growth factor mediated differentiation of a nerve cell line cultured in hormone-supplemental serum-free medium. *Dev. Brain Res.* 6:243–250.
- Hayashi, Y., and N. Miki. 1985. Purification and characterization of a neurite outgrowth factor from chicken gizzard smooth muscle. *J. Biol. Chem.* 260:14269–14275.
- Holley, R. W., and J. A. Kiernan. 1974. Control of the initiation of DNA synthesis in 3T3 cells: serum factors. *Proc. Natl. Acad. Sci. USA.* 71:2908–2912.

23. Kaiser, E. T., and F. J. Kezdy. 1984. Amphiphilic secondary structure: design of peptide hormones. *Science (Wash. DC)*. 223:249-255.
24. Kligman, D., and D. R. Marshak. 1985. Purification and characterization of a neurite extension factor from bovine brain. *Proc. Natl. Acad. Sci. USA*. 82:7136-7139.
25. Lander, A. D., D. K. Fujii, and L. F. Reichardt. 1985. Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. *J. Cell Biol.* 101:890-913.
26. Letourneau, P. C. 1986. Regulation of nerve fiber elongation during embryo-genesis. In *Developmental Neuropsychobiology*. W. T. Greenough and J. Juraska, editors. Academic Press Inc., New York. 33-51.
27. Levi-Montalcini, R., and P. U. Angeletti. 1968. Nerve growth factor. *Physiol Rev.* 48:534-569.
28. Ling, N., A. Baird, W. Wehrenberg, N. Ueno, T. Munegumi, and P. Brazeau. 1984. Synthesis and *in vitro* bioactivity of c-terminal deleted analogs of human growth hormone releasing factor. *Biochem. Biophys. Res. Commun.* 123:854-861.
29. Ling, N., F. Esch, P. Bohlen, P. Brazeau, W. Wehrenberg, and R. Guillemin. 1984. Isolation, primary structure and synthesis of human hypothalamic somatostatin growth hormone releasing factor. *Proc. Natl. Acad. Sci. USA*. 81:4302-4306.
30. Lobb, R. R., J. W. Harper, and J. W. Fett. 1986. Purification of heparin binding growth factors. *Anal. Biochem.* 154:1-14.
31. Manthorpe, M., E. Engvall, E. Ruoslahti, F. M. Longo, G. E. Davis, and S. Varon. 1983. Laminin promotes neuritic regeneration from cultured peripheral and central neurons. *J. Cell Biol.* 97:1882-1890.
32. Manthorpe, M., R. Fagnani, S. Skaper, and S. Varon. 1986. An automated colorimetric microassay for neuronotrophic factors. *Dev. Brain Res.* 25:191-198.
33. McKeenan, W. L. 1984. Use of basic polymers as synthetic substrata for cell culture. In *Methods for Preparation of Media, Supplements and Substrate for Serum Free Animal Cell Culture*. D. W. Barnes, D. A. Sirbasku, and G. H. Sato, editors. Alan R. Liss, New York. 209-213.
34. Merrifield, R. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149-2154.
35. Morgan, J. I., and W. Seifert. 1979. Growth factors and gangliosides: a possible new perspective in neuronal growth control. *J. Supramol. Struct.* 10:111-124.
36. Nishi, R., and D. Berg. 1981. Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in culture. *J. Neurosci.* 1:505-513.
37. O'Neill, C., P. Jordan, and G. Ireland. 1986. Evidence for two distinct mechanisms of anchorage stimulation in freshly explanted and 3T3 Swiss mouse fibroblasts. *Cell.* 44:489-496.
38. Otsuka, H., and M. Moskowitz. 1975. Anchorage dependent changes in transport of glucose, adenosine, uridine and leucine in 3T3 cells. *J. Cell. Physiol.* 86:379-388.
39. Ratner, N., R. P. Bunge, and L. Glaser. 1985. A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. *J. Cell Biol.* 101:744-754.
40. Ridout, D. C., M. Lambert, A. Kendall, G. Mae, D. Osterman, H. Tao, B. Weinstein, and E. Kaiser. 1985. Amphiphilic cationic peptides mediate cell adhesion to plastic surfaces. *J. Cell. Physiol.* 124:365-371.
41. Schubert, D. 1979. Early events after the interaction of NGF with sympathetic nerve cells. *Trends Neurosci.* 2:17-20.
42. Schubert, D. 1984. *Developmental Biology of Cultured Nerve Muscle and Glial*. Wiley Interscience, New York.
43. Schubert, D., and M. LaCorbiere. 1985. Isolation of an adhesion-mediating protein from chick neural retina adherens. *J. Cell Biol.* 101:1071-1077.
44. Schubert, D., and M. LaCorbiere. 1986. The role of purpurin in neural retina histogenesis. In *Proceedings of the Tenth International Congress of the International Society of Developmental Biologists*. H. C. Slavkin, editor. Alan R. Liss, Inc., New York. 3-16.
45. Schubert, D., S. Humphreys, F. DeVitry, and F. Jacob. 1971. Induced differentiation of neuroblastoma. *Dev. Biol.* 25:514-522.
46. Schubert, D., M. LaCorbiere, and F. Esch. 1986. A chick neural retina adhesion and survival molecule is a retinol-binding protein. *J. Cell Biol.* 102:2295-2301.
47. Schubert, D., M. LaCorbiere, and J. Watson. 1976. Growth regulation of cells grown in suspension culture. *Nature (Lond.)*. 264:266-267.
48. Steinberg, M. 1970. Does differential adhesion govern self-assembly processes in histogenesis? *J. Exp. Zool.* 173:395-433.
49. Stoker, M., C. H. O'Neill, S. Berryman, and V. Waxman. 1968. Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer.* 3:683-693.
50. Sutter, A., R. J. Riopelle, R. M. Harris-Warrick, and E. M. Shooter. 1979. Nerve growth factor receptors. *J. Biol. Chem.* 254:5972-5982.
51. Thomas, K. A., M. Rios-Candelore, and S. Fitzpatrick. 1984. Purification and characterization of acidic fibroblast growth factor from bovine brain. *Proc. Natl. Acad. Sci. USA*. 81:357-361.
52. Togari, A., G. Dickens, H. Ku-zyua, and G. Guroff. 1985. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* 5:307-316.
53. Walicke, P., M. Cowan, N. Ueno, A. Baird, and R. Guillemin. 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Natl. Acad. Sci. USA*. 83:3012-3016.